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RESEARCH PAPER

AT14A mediates the cell wall–plasma membrane–cytoskeleton continuum in *Arabidopsis thaliana* cells

Bing Lü[1], Juan Wang[1], Yu Zhang[1], Hongcheng Wang[1], Jiansheng Liang[1] and Jianhua Zhang[2,*]

[1] Key Laboratory of Crop Genetics and Physiology of Jiangsu Province, College of Bioscience and Biotechnology, Yangzhou University, Yangzhou, 225009 People's Republic of China
[2] School of Life Sciences and State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Hong Kong, China

* To whom correspondence should be addressed. E-mail: jhzhang@cuhk.edu.hk

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Abstract

AT14A has a small domain that has sequence similarities to integrins from animals. Integrins serve as a transmembrane linker between the extracellular matrix and the cytoskeleton, which play critical roles in a variety of biological processes. Because the function of AT14A is unknown, *Arabidopsis thaliana* AT14A, which is a transmembrane receptor for cell adhesion molecules and a middle member of the cell wall–plasma membrane–cytoskeleton continuum in plants, has been described. AT14A, co-expressed with green fluorescent protein (GFP), was found to localize mainly to the plasma membrane. The mutant *Arabidopsis at14a-1* cells exhibit various phenotypes with cell shape, cell cluster size, thickness, and cellulose content of cell wall, the adhesion between cells, and the adhesion of plasma membrane to cell wall varied by plasmolysis. Using direct staining of filamentous actin and indirect immunofluorescence staining of microtubules, cortical actin filaments and microtubules arrays were significantly altered in cells, either where AT14A was absent or over-expressed. It is concluded that AT14A may be a substantial middle member of the cell wall–plasma membrane–cytoskeleton continuum and play an important role in the continuum by regulating cell wall and cortical cytoskeleton organization.

Key words: *Arabidopsis thaliana*, AT14A, cell wall–plasma membrane–cytoskeleton continuum, transmembrane linker.

Introduction

AT14A is a membrane protein with unknown functions in *Arabidopsis thaliana*. Its cDNA was isolated by immunoscreening with an anti-integrin antibody from an expression library (Nagpal and Quatrano, 1999). The Blast analysis of the *AT14A* gene sequence showed that there were several similar sequences close to its locus on chromosome 3. *AT14A* should be a major member of the multigene family, although it had been thought to be a single copy gene by Nagpal and Quatrano. Analysis of the protein sequence showed that *AT14A* had an open reading frame encoding a protein of 385 amino acids and a predicted molecular weight of 43 kDa. AT14A possesses two transmembrane helices spanning amino acids, a very small outside part containing several amino acids, and two long inside regions with a small domain that has sequence similarities to integrins from fungi, insects, and humans, and is localized partly in the plasma membrane of *Arabidopsis* cells (Nagpal and Quatrano, 1999).

Integrins are a large family of heterodimeric transmembrane receptors for cell adhesion molecules in animal cells that are composed of non-covalently linked alpha and beta subunits, both of which typically comprise a short cytoplasmic tail (~20–50 residues), a single transmembrane helix, and a large extracellular domain (~700–1000 residues) (Wegener *et al.*, 2007). The large extracellular domains of integrins bind to the extracellular matrix (ECM), and the short cytoplasmic tails of integrin β subunits connect to the cytoskeleton. Besides serving as a transmembrane linker between ECM and cytoskeleton, integrins play both structural and bidirectional signal transducing roles (Boudreau and Jones, 1999; Qin *et al.*, 2004; Harburger and Calderwood, 2009; Legate *et al.*, 2009). Outside-in

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signalling informs the cell about the extracellular matrix environment, while inside-out signalling results in changes of integrin functional activity (Qin *et al.*, 2004). Hence integrin is involved in cell–cell, cell–matrix, and cell membrane–cytoskeleton interaction, and plays a particularly important role in cellular shape, adhesion, migration, and signal transduction in animals.

Some work suggests that the basic mechanisms of integrin function were very similar in animals and plants (Schindler *et al.*, 1989; Faik *et al.*, 1998; Blackman *et al.*, 2001; Clark *et al.*, 2001; Sakurai *et al.*, 2004; Gao *et al.*, 2007; Knepper *et al.*, 2011). Schindler *et al.* (1989) first reported the integrin-like protein in plants which is very similar to the integrins in animals. Since then, integrin-like protein has been identified by either immunological and/or biochemical methods in several plant species (Gens *et al.*, 1996; Canut *et al.*, 1998; Faik *et al.*, 1998; Labouré *et al.*, 1999; Laval *et al.*, 1999; Swatzell *et al.*, 1999; Garcia-Gomez *et al.*, 2000; Sun *et al.*, 2000; Sakurai *et al.*, 2004; Lü *et al.*, 2007*a*, *b*; Knepper *et al.*, 2011). Our previous results have also identified the presence of integrin-like protein in *Arabidopsis thaliana* and *Zea mays* plasma membrane, which mediates the interactions between the cell wall and the plasma membrane, and cell responses to osmotic stress (Lü *et al.*, 2007*a*, *b*). Although the analysis of the *Arabidopsis* genome sequence indicated that the sequences/genes encoding proteins which were high homologue to the integrins of animals do not exist (*Arabidopsis* Genome Initiative, 2000), there were plant proteins sharing similar motifs with animal integrins (Laval *et al.*, 1999; Clark *et al.*, 2001; Gentzbittel *et al.*, 2002; Knepper *et al.*, 2011). There existed some proteins in plants which may have a similar structure and function to integrins in animals.

In fact, accumulating evidence has shown that there is a cell wall–plasma membrane–cytoskeleton continuum in plant cells (Baluška *et al.*, 2003), and this continuum plays important roles in the regulation of plant responses to environmental cues (Zhou *et al.*, 2007). But its molecular basis mediated by the membrane protein is largely unknown. The sequence and structural similarity of AT14A to the integrins of animals suggests that AT14A may be one of the members of the cell wall–plasma membrane–cytoskeleton continuum and regulates signalling in plants. Plants, in a similar functional manner, must finely co-ordinate the cell wall and cytoskeleton, and carry out transmembrane signalling. In the present study, evidence is provided showing that AT14A functions like integrin in animals and mediates the cell wall–plasma membrane–cytoskeleton continuum.

Materials and methods

Plant growth conditions

The wild ecotype of *Arabidopsis thaliana* used in this study is Columbia (Col-0). The T-DNA insertion mutant *at14a-1* (SALK_101761) was obtained from ABRC. The primary callus cultures were raised from seeds. Established calli were grown on a half-strength Murashige and Skoog (MS) solidified medium containing 1 μg $\mathrm{ml^{-1}}$ 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 0.5 μg $\mathrm{ml^{-1}}$ kinetin at 24 °C in the dark and sub-cultured every 3–4 weeks. The cell lines were maintained in MS liquid medium containing 1 μg $\mathrm{ml^{-1}}$ 2, 4-D with shaking at 120 rpm, and were subcultured every week with 5% inoculums. The other growth conditions were the same during callus and cell suspension cultures.

Cloning, construction, and transformation

Total RNA was isolated from greenhouse-grown, 3–4-week-old wild-type *Arabidopsis* Col-0 leaves using the RNeasy® Plant Mini Kit (QIAGEN). First strand cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) and oligo(dT)18 as a primer. The full-length cDNA of *AT14A* was amplified by PCR. The PCR primer pair of *AT14A* is as follows: forward primer 5′-CACCATGGTGCTATCCAAA-3′, reverse primer 5′-TTTTCCAGAACCAGTGATCTT-3′. To ensure that no unintended mutations occurred during PCR, all constructs were identified or sequenced (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd) before transformation. The cloning and construction were made using Gateway technology (Invitrogen). Binary constructs used in this work were made using the pGWB5 vector (Invitrogen) containing the kanamycin and hygromycin resistance gene and the green fluorescent protein (GFP) reporter gene. The gene encoding *Arabidopsis* AT14A was inserted into the pGWB5 vector between the cauliflower mosaic virus 35S promoter and the *GFP* reporter gene. The resulting plasmid was used for transformation of *Agrobacterium tumefaciens* strain GV3101. *Arabidopsis thaliana* transformations were obtained by co-cultivating the suspended cells with *Agrobacterium*. All transgenic cell lines contain the *AT14A: GFP* or the *GFP* reporter only.

Immunoblot analysis

Crude extracts were prepared from *Arabidopsis* callus cells. About 500 mg (fresh weight) calli were ground in 500 μl of extraction buffer [20 mmol $\mathrm{l^{-1}}$ TRIS-HCl, pH 8.0, 5 mmol $\mathrm{l^{-1}}$ ethylenediaminetetraacetic acid (EDTA), 1 mmol $\mathrm{l^{-1}}$ phenylmethylsulphonyl fluoride (PMSF), 5 mmol $\mathrm{l^{-1}}$ ethyleneglycol-*bis*(2-aminoethoxy)-tetraacetic acid (EGTA), 10 mmol $\mathrm{l^{-1}}$ dithiothreitol (DTT), and 0.05% sodium dodecyl sulphate (SDS)] and centrifuged at 20 000 *g* for 20 min to remove debris.

Total proteins prepared as described above were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% gels. For immunoblot analysis, the proteins were transferred to polyvinylidene difluoride membranes. These membranes were then blocked with 5% dry milk in TBST (50 mmol $\mathrm{l^{-1}}$ TRIS-HCl, pH 7.5, 150 mmol $\mathrm{l^{-1}}$ NaCl, and 0.05% Tween-20) for 1 h at 37 °C, and then incubated with the first antibody against GFP (rabbit IgG anti-GFP, Epitomics, E-385) at a dilution of 1:10 000 in 5% dry milk-TBST for O/N at 4 °C. The second antibody used was a horseradish peroxidase-linked anti-rabbit antibody with a 1:10 000 dilution. After three 10 min washes with shaking, development was accomplished by Roche™ Lumi-Light Western blot Substrate for 30 s following the manufacturer's instructions, and the membrane was exposed to Kodak X-ray film. The results were obtained through Kodak medical X-ray processor 102 (Eastman Kodak, Rochester, USA).

Fluorescence microscopy

The transgenic cell lines were grown in MS for 4 d. Specimens were observed using an Olympus BX51 fluorescence microscope with a GFP filter. A ×40 objective was used to scan the samples. GFP signals were false coloured green.

Phenotype analysis

Quantitative analysis of the different cellular shape: The cell lines used in the phenotype analysis were observed on the fourth day after subculture. The cells were classified into three groups according to their shape (acerose cells, oval cells, and round cells) with a light microscope. The numbers of different shapes of the suspended cells of three genotypes were each counted.

Quantitative analysis of the cell cluster size: The cell clusters in suspended cells were classified into three groups according to the cell numbers per cell cluster, i.e. 1 cell per cell cluster, 2–10 cells per cell cluster, and >10 cells per cell cluster. The numbers of clusters of different sizes of the suspended cells of three genotypes were counted on the fourth day after subculture.

Cell wall thickness measurement: The cell wall images of the suspended cells of three genotypes were obtained with the ×100 oil-immersion objective of light micrographs. Using the imaging software (Image J 1.37V), the cell wall thicknesses of the suspended cells of the different genotypes were measured. For each sample, cell wall thicknesses were measured over 100 cells.

Measurement of cellulose content: For each sample, 0.5–0.9 g calli were ground, incubated in 70% ethanol at 70 °C and then incubated in 100% acetone at room temperature for 5 min. The percentage of cellulose in the dry weight of calli was evaluated according to the (modified) method described by Updegraff (1969). Ground calli were incubated at 100 °C with nitric acid/acetic acid/water solution (1:8:2, by vol.) for 30 min. After centrifugation at 3000 *g* for 60 min at room temperature, a solution of 60% sulphuric acid was added to the precipitate. To evaluate the percentage of cellulose, the solution was mixed with 2 ml of sample, 0.5 ml of 2% anthrone solution, and 5 ml of sulphuric acid. The spectrophotometric measurement was made against a cellulose curve to 620 nm.

Plasmolysis

Cells were treated as described by Lü *et al.* (2007*a*, *b*). For hyperosmotic treatment, cells were incubated in a hyperosmotic solution with 722 mmol kg^{-1} of osmolality (a mixture of glucose and sorbitol, 230 mmol kg^{-1} of osmolality as a control) for 6 h. The plasmolyses were observed with a light microscope and the plasmolysis index was calculated by the following equation:

$$\text{Plasmolysis index} = (N_0{\times}0 + N_1{\times}1 + N_2{\times}2 + N_3{\times}3 + N_4{\times}4)/(N_0{+}N_1{+}N_2{+}N_3{+}N_4)$$

where 0, 1, 2, 3, and 4 represent no plasmolysis, less than 25% plasmolysis, 26–50% plasmolysis, 51–75 plasmolysis, and 76–100% plasmolysis, respectively, and N_0, N_1, N_2, N_3, and N_4 represent the numbers of cells plasmolysed to each degree in each view.

Statistical analysis

The significance analyses were carried out using the SAS statistical analysis package (version 6.12, SAS Institute, Cary, NC, USA). Multiple comparisons were made among different treatments from at least three repeat experiments. Data were accepted as statistically significant at the 0.05 level.

Fluorescence localization of actin and β-tubulin

Fluorescence localization of actin: The filamentous actin cytoskeleton was visualized by treating a culture of *Arabidopsis* cells with 10 nmol l^{-1} Alexa Fluor® 488 phalloidin in PEM [50 mmol l^{-1} PIPES, pH 6.9, 5 mmol l^{-1} EGTA, 5 mmol l^{-1} $MgSO_4$, 0.4 mol l^{-1} mannitol, 2% dimethyl sulphoxide (DMSO), and 0.02% NP-40] for 10 min.

Immunofluorescence localization of β-tubulin: The main procedure for the immunofluorescence localization of β-tubulin has been described previously by Lü *et al.* (2007*c*). Suspended cells of *Arabidopsis* were harvested and fixed at room temperature. Those cells were then enzymatically digested in PBS buffer containing 1% (w/v) cellulase and 0.5% (w/v) pectinase at 37 °C for 30 min. Cells were treated with Triton X-100, before being incubated in PBS buffer containing 1% (w/v) bovine serum albumin (BSA). The cells were then incubated with a primary antibody against β-tubulin produced in mouse (T4026, Sigma, St Louis, MO, USA) at a dilution of 1:500 at 37 °C for 2 h. After washing three times with PBS, cells were incubated with TRITC-conjugated anti-mouse immunoglobulin (T7657, Sigma) at a dilution of 1:400 at 37 °C for 2 h. Specimens were mounted in 2% DABCO (1, 4-diazabicyclo [2,2,2,]octane; D-2522, Sigma).

Laser scanning confocal microscopy: Images were collected with a ZEISS® LSM510 confocal microscope. Alexa Fluor® 488 phalloidin emission was detected using a 488 nm band-pass filter. TRITC was excited with a 543 nm laser line. The images were coded green (Alexa 488) and magenta (TRITC), respectively. A ×100 oil-immersion objective was used to scan the samples. Each image shown represents either a single focal plan or a projection of individual images taken as a Z series. To determine the specificity of the signals, sequential scans were performed.

Results

AT14A protein mainly localizes in plasma membrane

To investigate the biological activity of *AT14A* transformants, the expression and localization of AT14A–GFP fusion protein was first studied using immunoblotting analysis and fluorescence microscopy in *Arabidopsis* cells (Fig. 1). The total proteins from *Arabidopsis* calli were incubated with an antibody against GFP. As shown in Fig. 1A, a specific protein band with a molecular mass of around 70 kDa was detected in *AT14A–GFP*-over-expressed transformants by immunoblot analysis, but not in the wild type and the *at14a* mutant. To ascertain the subcellular localization of AT14A, the localization of GFP in transforming *Arabidopsis* suspended cells was also studied (Fig. 1B). The GFP fluorescence was detected mainly in the plasma membrane of *AT14A–GFP*-over-expressed suspended cells. As a control, suspended cell lines of the wild type only over-expressing a *GFP* reporter gene were also established and the GFP was detected not only in the plasma membrane but all over the cytoplasm. As an additional control, suspended cell lines of the mutant *at14a-1* expressing an AT14A–GFP fusion protein were also established. The GFP fluorescence was also detected in the plasma membrane, while the GFP was detected all over the cytoplasm in mutant cells only expressing a GFP protein derived by the *35S* promoter. No GFP fluorescence was detected in Columbia and the mutant *at14a-1* (data not shown). These results clearly indicated that AT14A protein is a membrane protein.

AT14A protein mediates cell shape, cell-to-cell adhesion and cell wall formation

To study the functions of AT14A protein further, the cell shape of different genotypes, i.e. the wild-type Columbia, the *at14a-1* mutant, and *AT14A*-over-expressed transformants was compared first using suspension-cultured cell lines (Fig. 2A, B). More than 95% of cells in the wild type are

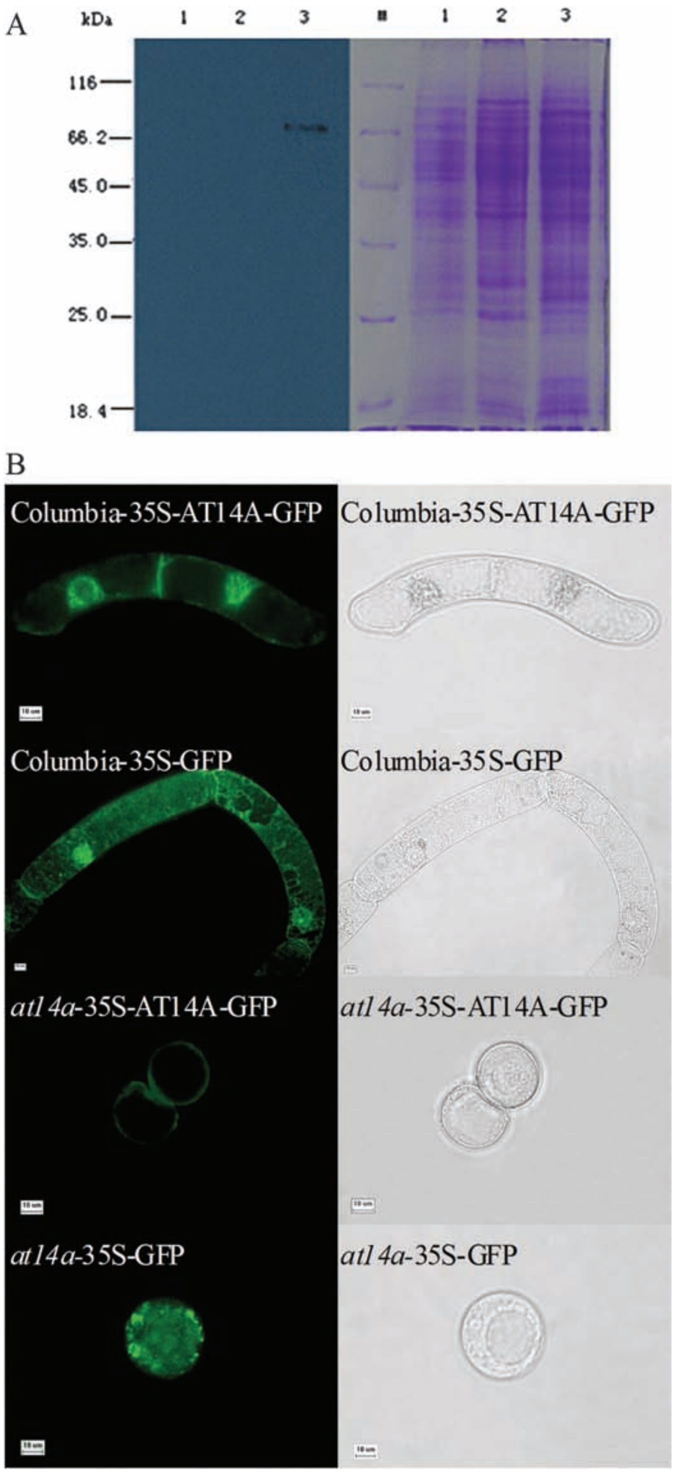

Fig. 1. Cellular localization of AT14A proteins. (A) Immunoblot analysis of AT14A-GFP fusion protein in calli of *Arabidopsis*. Proteins isolated and purified from wild-type (Columbia, Lane 1), mutant (*at14a-1*, Lane 2), and over-expressed calli (*AT14A*, Lane 3) were separated on SDS-PAGE, and probed with an antibody against GFP. The molecular weight of protein specifically reacted with GFP antibody is about 70 kDa. (B) The GFP fluorescence was detected using an Olympus BX51 fluorescence microscope. Left: fluorescent field, and Right: bright field of the corresponding cells (Bar=10 μm).

acerose or oval, and the cells stick to each other and form stringlike cell chains. The cells of the *at14a-1* mutant exhibit a characteristic round or oval shape and almost no cell clusters are formed, while most of the *AT14A* over-expressed cells show an oval shape and the cells form large cell clusters usually containing over 10 cells per cell cluster (Fig. 2C).These results suggest that the AT14A protein regulates cell shape and cell-to-cell adhesion.

Figure 2D showed the differences in cell wall thickness among three different genotypes. Compared with the wild type, *at14a-1* mutant cells had the thinnest cell wall, while *AT14A*-over-expressed cells had a thicker cell wall than the *at14a-1* mutant cells but a thinner cell wall than the wild type.

Compositional analysis of cell walls isolated from *Arabidopsis* calli of three different genotypes revealed substantial differences in cellulose content among them (Fig. 2E). As in the case of cell wall thickness, the wild-type cell wall had a higher cellulose content than the *at14a-1* mutant cell wall. Surprisingly, the cell wall of *AT14A*-over-expressed transformants had the highest cellulose content, which was significantly higher than wild-type cell walls. Understandably, cell wall biosynthesis and the deposition of cell wall substances were very complicated and well-regulated and the roles of AT14A may not cover all the aspects of cell wall biosynthesis and secretion of cell wall substances.

AT14A alters the adhesion of the plasma membrane to the cell wall

The sequence and structural similarity of AT14A to the integrins of animals led us to examine a physiological role for AT14A in maintaining cell integrity through plasma membrane–cell wall adhesions. The suspended cells of the three genotypes were visualized before and after osmotic stress-induced plasmolysis to assess the integrity of plasma membrane–cell wall adhesion (Fig. 3A). When suspended cells were incubated in the same hyperosmotic solution (a mixture of glucose and sorbitol with an osmotic potential of 722 mmol kg^{-1}) for 6 h, the plasma membrane separated from the cell wall, but the extent of plasmolysis was dependent on the different genotypes of *Arabidopsis* (Fig. 3). As shown in Fig. 3A, an obvious attachment of plasma membrane to the cell wall was visible in wild-type cells and the plasmolysis index was 0.23. In mutant cells, more severe plasmolyses were observed. The plasma membrane detached from the cell wall at most regions and the plasmolysis index nearly reached 0.5 (Fig. 3B). Over-expression of AT14A had no obvious effect on the hyperosmotic stress-induced plasmolysis compared with the wild-type cells.

AT14A protein is closely related to the arrangement of microfilaments and microtubules

Our results clearly indicated that At14A protein controlled cell shape and the adhesion of plasma membrane to the cell wall, as well as cell wall biosynthesis. It is well known that

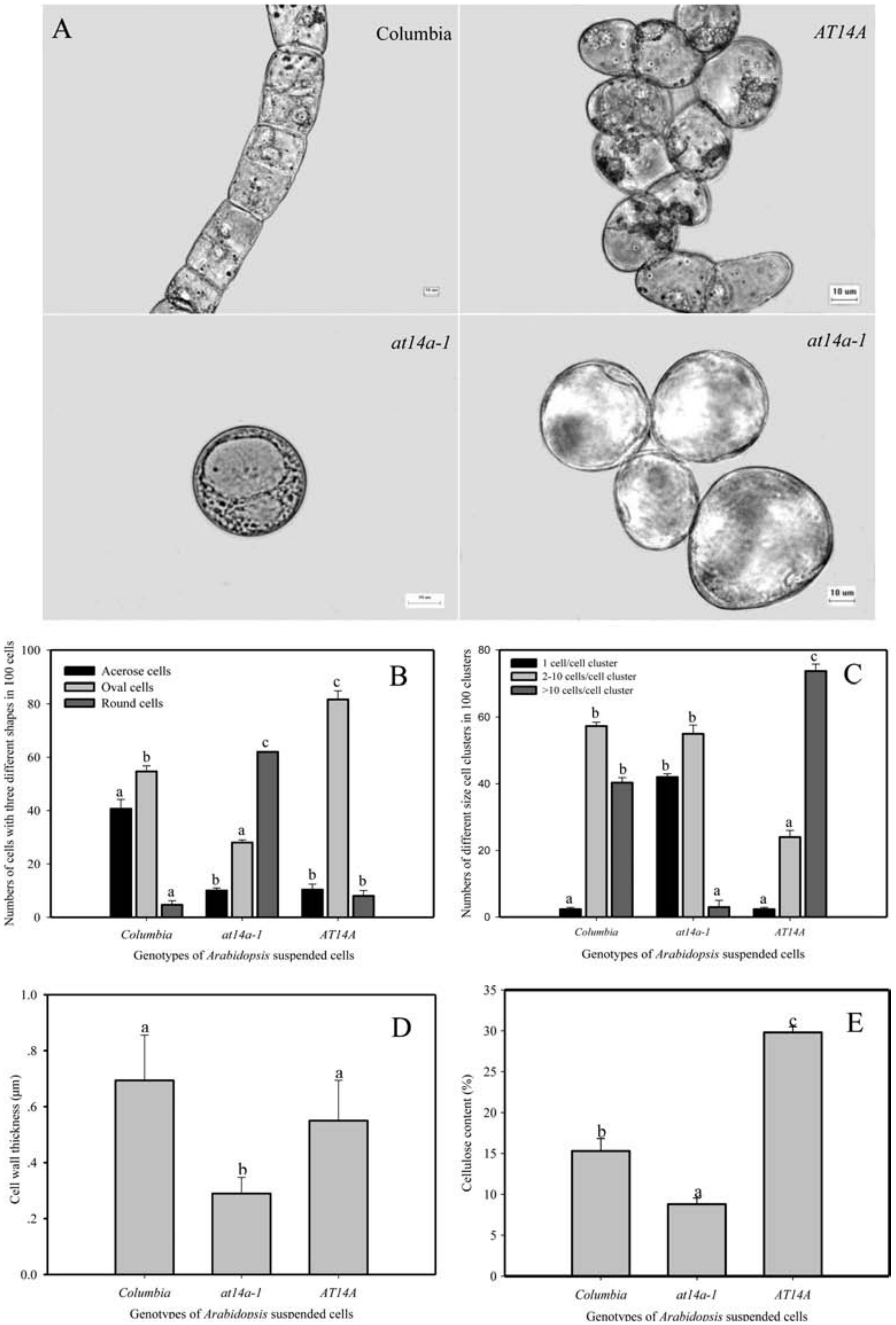

Fig. 2. Phenotypes of the suspended cells of the three genotypes. (A) The cells in a suspension culture under the microscope: Columbia (wild type), *AT14A* (over-expressed type), and *at14a-1* (null mutant) (bar=10 μm). (B) Quantitative analysis of the different shapes of the suspended cells of the three genotypes. (C) Quantitative analysis of the cell clusters size of the cell lines of the three genotypes. (D) Thickness of the cell wall of suspended cells of the three genotypes of *Arabidopsis*. Mean thicknesses are given ±SE (n=100) from three separate experiments. (E) Cellulose contents of the cells of the three genotypes. Data are means of three separate experiments ±SE. Different letters represent significant differences between different genotypes at the 0.05 level.

cell shape is established under the control of the cytoskeleton that is composed of microfilaments, intermediate filaments, and microtubules. Therefore, it is reasonable to assume that, if AT14A protein is indeed involved in the regulation of cell shape, there may be interactions between the AT14A protein and the cytoskeleton. Here, the differences in the arrangement of the microfilaments and microtubules are compared among the three different genotypes

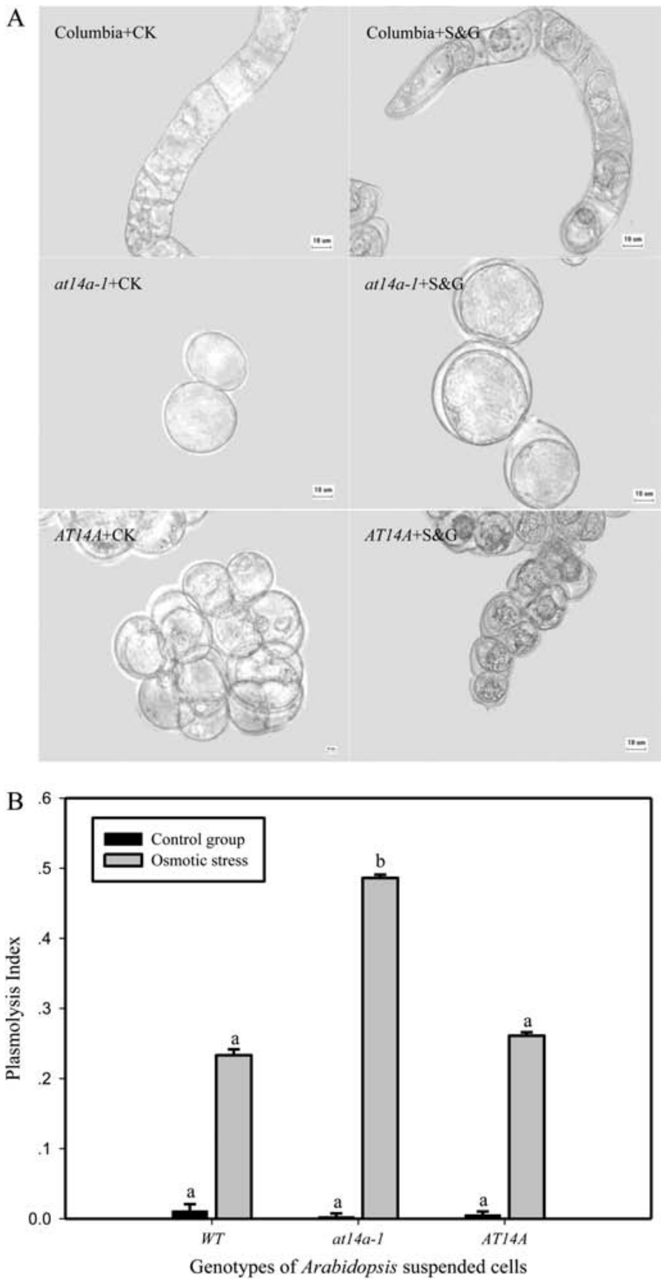

Fig. 3. AT14A altered the plasmolysis and the adhesion of the plasma membrane to the cell wall. (A) Plasmolysis induced by hyperosmotic stress in suspended cells of the three different genotypes: Columbia (wild type); the *at14a-1* (null mutant), and *AT14A* (over-expressed type). CK, cells were incubated in a control medium; S&G, cells were incubated in a hyperosmotic sorbitol and glucose solution with 722 mmol kg^{-1} of osmolality for 6 h. Scale bar=10 μm. (B) Plasmolysis index analysis of suspended cells of the three different genotypes of *Arabidopsis* treated with osmotic stress (described above). The degree of plasmolysis was observed under a light microscope. More than 30 cells were observed in each view and the plasmolysis index was calculated as described in the ‘Materials and methods’. Data are means of three separate experiments ±SE. Different letters represent significant differences between different genotypes at the 0.05 level.

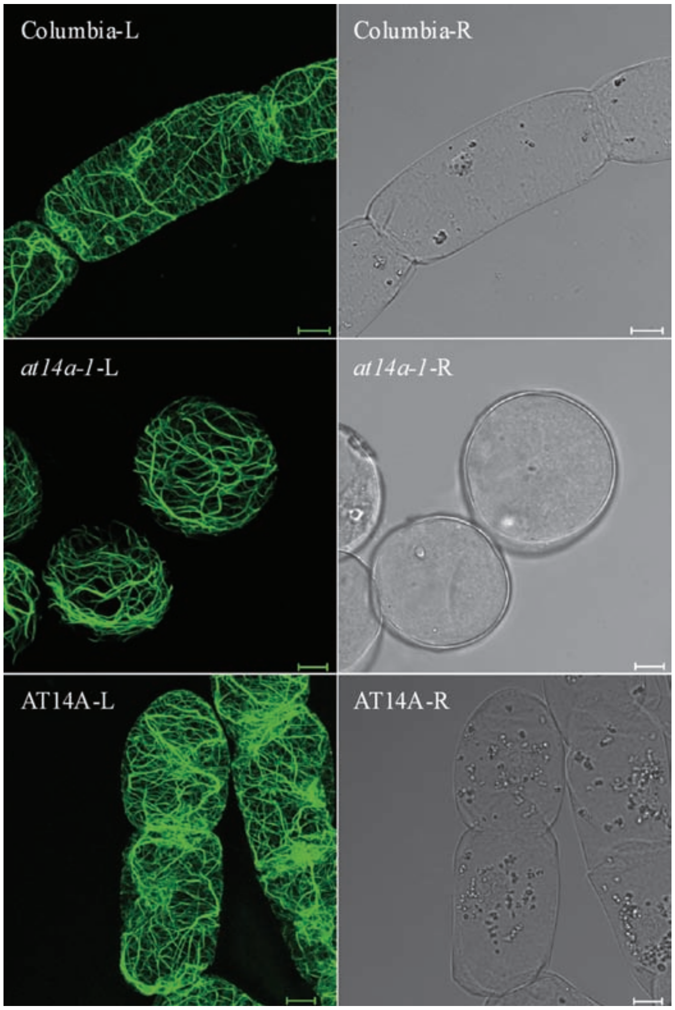

Fig. 4. Fluorescent images of microfilaments of the suspended cells of the three genotypes: Columbia (wild type), the *at14a-1* (null mutant), and *AT14A* (over-expressed type); (right) the cell labelled with the Alexa Fluor® 488 phalloidin showing the actin; (left) the same cell as on the right but as bright field images (bar=10 μm).

used in the present study. Figure 4 showed the microfilament arrangement of the three genotypes observed by direct staining with Alexa Fluor® 488 phalloidin. The microfilaments in the wild-type cells lay in ordered arrays, most of them transverse to the long axis of the cells. A similar microfilament arrangement, but a much denser one was observed in the cells of AT14A-over-expressed transformants. A significant difference in microfilament arrangement was observed in the *at14a* null mutant cells, in which the microfilament arrangement was much more random, sparser, and thicker (Fig. 4).

The cortical microtubules were visualized by indirect immunofluorescence staining with anti-tubulin antibodies (Fig. 5). The cortical microtubules were arranged into a fine structure with a predominant orientation parallel to the short axis of the cells in the wild-type cells and in the cells of the AT14A-over-expressed transformants, whereas more random and sparse arrangement of microtubules was observed in the *at14a-1* mutant cells.

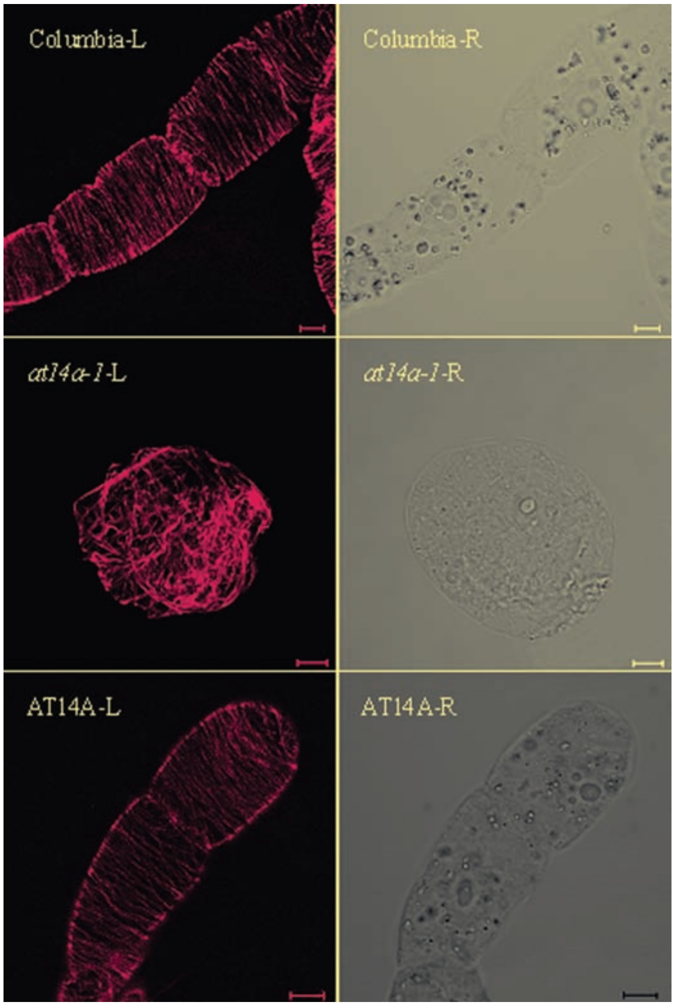

Fig. 5. Fluorescent images of microtubules of the suspended cells of the three genotypes: Columbia (wild type), the *at14a-1* (null mutant), and *AT14A* (over-expressed type); (right) the cell labelled with the TRITC-conjugated antibody showing the β-tubulin; (left) the same cell as on the right but as bright field images (bar=10 μm).

Discussion

Co-ordination between the sites for cell division and expansion as well as the responses to external environmental cues requires some type of communication, possibly through signalling cascades that are triggered by directional cues from internal and/or external sources. It has been recognized that the interactions between the cytoskeleton, the plasma membrane, and the cell wall during plant cell morphogenesis are of importance. However, the nature of the macromolecular complexes linking cell wall morphogenesis with the cytoskeleton is largely unknown in plant cells. In animal cells, the regulation of their morphology, migratory properties, growth, and differentiation are largely through their adhesive interaction with the extracellular matrix (ECM), which is mediated by integrins. Integrins are transmembrane proteins that serve as receptors for ECM components and provide attachment domains for bundles of actin filaments through actin-binding proteins. Several pieces of evidence have indicated that interaction between cytoskeletal systems and the extracellular matrix (such as the cell wall) also occurs in plant cells (Baluška *et al.*, 2003; Martiniere *et al.*, 2011). Understandably, it is most important to explore the nature and roles of the molecules involved in the regulation of interaction between the cytoskeleton, the plasma membrane, and the cell wall. Several actin interacting proteins have been postulated as the physical link between the cell wall and the actin cytoskeleton (Baluška *et al.*, 2003). In the present study, it is reported that AT14A protein, a plasma membrane protein, may act as the integrins in animal cells and be involved in the regulation of cytoskeleton arrangement, cell wall biosynthesis, and plasmolysis under osmotic stress.

AT14A was identified more than 10 years ago (Nagpal and Quatrano, 1999) by immuoscreening from an *Arabidopsis* expression library with an anti-integrin antibody. Computer prediction showed that the AT14A protein is a plasma membrane-localized protein and possesses two transmembrane domains, which was proved by the results of immunoblotting and subcellular localization of the AT14A–GFP fusion protein (Fig. 1). The cell shape and cell-to-cell adhesion was also analysed using suspension-cultured cell systems with different expression levels of the *At14A* gene to explore whether the AT14A protein is involved in controlling cell shape and cell to cell adhesion. Our results clearly indicated that AT14A regulated cell shape and cell-to-cell adhesion (Fig. 2), although the molecular mechanisms are not very clear. The suspended cells of the mutant *at14a-1* exhibit a characteristic round phenotype and an absence of cell-to-cell adhesion, which was very similar to the cases in animal cells (Chen *et al.*, 2003; Yeung *et al.*, 2005). As it is known, the shape of plant cells is controlled largely by the cell wall outside cell and cytoskeleton inside cell (Wymer and Lloyd, 1996). The cell wall, the plasma membrane, and the cytoskeleton are considered to be the main actors in the establishment of polarity and morphogenesis of the cells (Laval *et al.*, 1999; Lecuit and Pilot, 2003). Therefore, it is suggested that AT14A might be involved in the mediation of interactions between the cell wall and plasma membrane, and/or the plasma membrane and cytoskeleton (Qin *et al.*, 2004; Legate *et al.*, 2009; Harburger and Calderwood, 2009).

Further evidence on the involvement of AT14A in the interaction between the cell wall and plasma membrane came from the studies that AT14A controls the cell wall biosynthesis (Fig. 2D, E) and plasmolysis under osmotic stress (Fig. 3). Our results showed that AT14A positively controlled cell wall thickness, largely as a result of the stimulation of cellulose biosynthesis, although there is little information at present about how AT14A controls cellulose biosynthesis and the relationship between the cortical microtubules and cellulose biosynthesis. Thus, AT14A might provide an integrin-like function in plants and be linked to the deposition and alignment of cellulose microfibrils (Andrawis *et al.*, 1993; Delmer and Amor, 1995; Calvert *et al.*, 1996). Using a cell biology-based approach, combined with the osmotic stress treatment, a role for AT14A in mediating plasma membrane–cell wall adhesions has also been identified (Fig. 3). The differences in plasmolytic patterns in various cell types suggested the changes of the linkages between the components of the cell wall–plasma membrane–cytoskeleton continuum (Wojtaszek *et al.*, 2007). Therefore, it is reasonably to assume that AT14A may be one of the central components in the cell wall–plasma membrane–cytoskeleton continuum.

Plant cell expansion and morphology are mainly controlled by the spatial organization of cortical microtubule arrays and plant cells might use an active process to generate transverse microtubule arrays, which are essential for controlling the direction of cell, tissue, and organ growth. Several microtubule-associated proteins (such as MOR1, CLASP, MAP65s, and katanins) have been identified to be crucial for fine-tuning the organization of microtubule arrays (see the review by Wasteneys and Ambrose, 2009). The above discussion clearly showed that AT14A regulated cell morphology and cell-to-cell adhesion, but understanding of whether and how the AT14A interacts with cytoskeletons remained to be established. However, a comparison of the filamentous actin and cortical microtubule arrays in suspension-cultured cell systems with different genetic backgrounds showed significant differences in the spatial organization of the actin and microtubule cytoskeletons (Figs 4, 5), which implied that AT14A might interact with cytoskeletons.

Taken together, AT14A is an essential core component of the cell wall–plasma membrane–cytoskeleton continuum. The key function of AT14A may be to connect the cell wall to the cytoskeleton (filamentous actins or cortical microtubule). AT14A serves as a transmembrane linker between the cell wall and the cytoskeleton in plants just as integrin in animals, and plays important roles in controlling polarity and morphogenesis.

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References

Andrawis A, Solomon M, Delmer DP. 1993. Cotton fiber annexins: a potential role in the regulation of callose synthase. *The Plant Journal* **3,** 763–772.

***Arabidopsis* Genome Initiative.** 2000. Analysis of the genome sequence of the flowering plant. *Arabidopsis thaliana. Nature* **408,** 796–815.

Baluška F, Šamaj J, Wojtaszek P, Volkmann D, Menzel D. 2003. Cytoskeleton-plasma membrane-cell wall continuum in plants. Emerging links revisited. *Plant Physiology* **133,** 482–491.

Blackman SA, Miedema M, Yeung EC, Staves MP. 2001. Effect of the tetrapeptide RGDS on somatic embryogenesis in *Daucus carota. Physiologia Plantarum* **112,** 567–571.

Boudreau NJ, Jones PL. 1999. Extracellular matrix and integrin signalling: the shape of things to come. *Biochemical Journal* **339,** 481–488.

Calvert CM, Gant SJ, Bowles DJ. 1996. Tomato annexins p34 and p35 bind to F-actin and display nucleotide phosphodiesterase activity inhibited by phospholipid binding. *The Plant Cell* **8,** 333–342.

Canut H, Carrasco A, Galaud JP, Cassan C, Bouyssou H, Vita N, Ferrara P, Pont-Lezica R. 1998. High affinity RGD binding sites at the plasma membrane of *Arabidopsis thaliana* links the cell wall. *The Plant Journal* **16,** 63–71.

Chen CS, Alonso JL, Ostuni E, Whitesides GM, Ingber DE. 2003. Cell shape provides global control of focal adhesion assembly. *Biochemical and Biophysical Research Communications* **307,** 355–361.

Clark GB, Thompson Jr G, Roux SJ. 2001. Signal transduction mechanisms in plants: An overview. *Current Science* **80,** 170–177.

Delmer DP, Amor Y. 1995. Cellulose biosynthesis. *The Plant Cell* **7,** 987–1000.

Faik A, Laboure AM, Gulino D, Mandaron P, Falconet DA. 1998. A plant surface protein sharing structural properties with animal integrins. *European Journal of Biochemistry* **253,** 552–559.

Gao H, Gong YW, Yuan YJ. 2007. RGD-dependent mechanotransduction of suspension cultured *Taxus* cell in response to shear stress. *Biotechnology Progress* **23,** 673–679.

Garcia-Gomez BI, Campos F, Hernandez M, Covarrubias AA. 2000. Two bean cell wall proteins more abundant during water deficit are high in proline and interact with a plasma membrane protein. *The Plant Journal* **22,** 277–288.

Gens JS, Reuzeau C, Doolittle KW, McNally JG, Pickard BG. 1996. Covisualization by computational optical-sectioning microscopy of integrin and associated proteins at the cell membrane of living onion protoplasts. *Protoplasma* **194,** 215–230.

Gentzbittel L, Abbott A, Galaud JP, Georgi F, Fabre F, Liboz T, Alibert G. 2002. A bacterial artificial chromosome (BAC) library for sunflower, and identification of clones containing genes for putative transmembrane receptors. *Molecular Genetics and Genomics* **266,** 979–987.

Harburger DS, Calderwood DA. 2009. Integrin signaling at a glance. *Journal of Cell Science* **122,** 159–163.

Knepper C, Savory EA, Day B. 2011. Arabidopsis NDR1 is an integrin-like protein with a role in fluid loss and plasma membrane-cell wall adhesion. *Plant Physiology* **156,** 286–300.

Labouré AM, Faik A, Mandaron P, Falconet D. 1999. RGD-dependent growth of maize calluses and immunodetection of an integrin-like protein. *FEBS Letters* **442,** 123–128.

Laval V, Chabannes M, Carrière M, Canut H, Barre A, Rougé P, Pont-Lezica R, Galaud J. 1999. A family of Arabidopsis plasma membrane receptors presenting animal β-integrin domains. *Biochimica et Biophysica Acta* **1435,** 61–70.

Lecuit T, Pilot F. 2003. Developmental control of cell morphogenesis: a focus on membrane growth. *Nature Cell Biology* **5,** 103–108.

Legate KR, Wickström SA, Fässier R. 2009. Genetic and cell biological analysis of integrin outside-in signaling. *Genes and Development* **23,** 397–418.

Lü B, Chen F, Gong ZH, Xie H, Liang JS. 2007*a*. Integrin-like protein is involved in the osmotic stress-induced abscisic acid biosynthesis in *Arabidopsis thaliana*. *Journal of Integrative Plant Biology* **49,** 540–549.

Lü B, Chen F, Gong ZH, Xie H, Zhang JH, Liang JS. 2007*b*. Intracellular localization of integrin-like protein and its roles in osmotic stress-induced ABA biosynthesis in. *Zea mays. Protoplasma* **232,** 35–43.

Lü B, Gong ZH, Wang J, Zhang JH, Liang JS. 2007*c*. Microtubule dynamics in *Zea mays* roots in response to drought stress. *Journal of Experimental Botany* **58,** 2565–2572.

Martiniere A, Gayral P, Hawes C, Runions J. 2011. Building bridges: formin1 of Arabidopsis forms a connection between the cell wall and the actin cytoskeleton. *The Plant Journal* **66,** 354–365.

Nagpal P, Quatrano RS. 1999. Isolation and characterization of a cDNA clone from *Arabidopsis thaliana* with partial sequence similarity to integrins. *Gene* **230,** 33–40.

Qin J, Vinogradova O, Plow EF. 2004. Integrin bidirectional signaling: a molecular view. *PLoS Biology* **2,** 726–729.

Sakurai M, Pak JY, Muramatsu Y, Fukuhara T. 2004. Integrin-like protein at the invaginated plasma membrane of epidermal cells in mature leaves of the marine angiosperm *Zostera marina* L. *Planta* **220,** 271–277.

Schindler M, Meiners S, Cheresh DA. 1989. RGD-dependent linkage between plant cell wall and plasma membrane: consequences for growth. *Journal of Cell Biology* **108,** 1955–1965.

Sun Y, Qian H, Xu XD, Han Y, Yen L, Sun D. 2000. Integrin-like proteins in pollen tube: detection, localization and function. *Plant and Cell Physiology* **41,** 1136–1142.

Swatzell LJ, Edelmann RE, Makaroff CA, Kiss JZ. 1999. Integrin-like proteins are localized to plasma membrane fractions, not plastids, in *Arabidopsis. Plant and Cell Physiology* **40,** 173–183.

Updegraff DM. 1969. Semi-micro determination of cellulose in biological materials. *Analytical Biochemistry* **32,** 420–424.

Wasteneys GO, Ambrose JC. 2009. Spatial organization of plant cortical microtubules: close encounters of the 2D kind. *Trends in Cell Biology* **19,** 62–71.

Wegener KL, Partridge AW, Han J, Pickford AR, Liddington RC, Ginsberg MH, Campbell LD. 2007. Structural basis of integrin activation by talin. *Cell* **128,** 171–182.

Wojtaszek P, Baluška F, Kasprowicz A, Luczak M, Volkmann D. 2007. Domain-specific mechanosensory transmission of osmotic and enzymatic cell wall disturbances to the actin cytoskeleton. *Protoplasma* **230,** 217–230.

Wymer C, Lloyd C. 1996. Dynamic microtubules: implications for cell wall patterns. *Trends in Plant Science* **1,** 222–228.

Yeung T, Georges PC, Flanagan LA, Marg B, Ortiz M, Funaki M, Zahir N, Ming W, Weaver V, Janmey PA. 2005. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motility and the Cytoskeleton* **60,** 24–34.

Zhou J, Wang B, Li Y, Wang Y, Zhu L. 2007. Responses of chrysanthemum cells to mechanical stimulation require intact microtubules and plasma membrane–cell wall adhesion. *Journal of Plant Growth Regulation* **26,** 55–68.